Communications to the editor

A METHOD FOR MEASURING THE OUTER MEMBRANE-PERMEABILITY OF β-LACTAM ANTIBIOTICS IN GRAM-NEGATIVE BACTERIA

Sir:

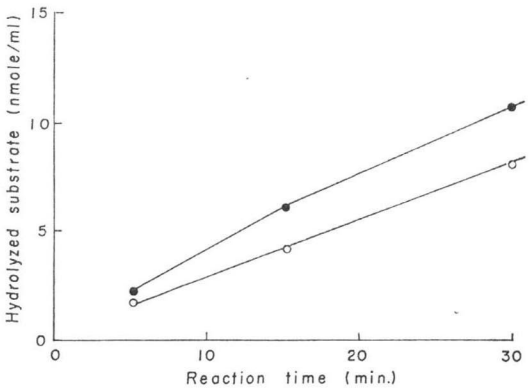
Outer membrane of gram-negative bacteria is the barrier which disturbs penetration of β-lactam antibiotics into their targets located on inner membrane. The ability of the antibiotics to pass through outer membrane is one of the important properties influencing upon their antibacterial activity and spectrum. However, a direct method for assaying the antibiotic-concentration achieved around the targets has not been known. Recently, we devised a method* for estimating the antibiotic-concentration in the periplasm which is the space around the targets. The present communication reports the application of the method to the *Proteus morganii* cells with five β-lactam antibiotics, *i.e.*, benzylpenicillin, ampicillin, cephalothin, cefazolin and cephalexin.

The principle of the method is as follows: β-Lactamase of gram-negative bacteria is located in the periplasm. When intact cells are used as the β-lactamase sample and the enzyme reaction is carried out at the substrate concentrations lower than the *Km* value of the enzyme for the substrate, the reaction velocity may be proportional to the substrate concentration achieved in the periplasm. With the enzyme activity of a bacterial cell suspension before and after disruption, the *Km* value (μM) and the substrate concentration in the reaction medium (S_1 μM), the substrate concentration in the periplasm (S_1 μM) is estimated from a following formula which is derived from the MICHAELIS-MENTEN equation. The symbol C is the ratio of *Vi* to *Vd*. Where, *Vi* and *Vd* are defined as the enzyme activities of a bacterial cell suspension before and after disruption, respectively.

$$S_2 = \frac{1}{C}\left(\frac{Km \cdot S_1}{Km + S_1 - S_1/C}\right)$$

The tested organism, *P. morganii* 1510, is a clinical isolate and produces a cephalosporinase constitutively.[1] The *Km* values of the enzyme for benzylpenicillin, ampicillin, cephalothin, cefazolin and cephalexin are 426, 90, 12, 28 and 14 μM, respectively. The bacterial cells growing exponentially in heart infusion broth at 37°C were harvested by centrifugation and washed once with 13 mM phosphate buffer containing 0.68% NaCl and 1 mM $MgSO_4$ (pH 7.0), and then resuspended in the buffer. The cell suspension was divided into two portions. One was used as the intact cell sample and another as the disrupted cell sample after treated with an ultrasonic disintegrator for 2 minutes. The intact cells were kept at 25°C during handling, and used as the enzyme sample within one hour. The iodometric assay method of PERRET[2] was employed for the β-lactamase assay at substrate concentration of 8 mM. For lower substrate concentrations, the microiodometric method devised by NOVICK[3] was used with modification. The enzyme reaction was carried out in the buffer mentioned above at 30°C for 15 minutes, and the consumed substrate during the enzyme reaction was less than 20% of the substrate added. It should be emphasized that

Fig. 1. Kinetics of benzylpenicillin-, cefazolin-hydrolysis by the intact cells.
The enzyme reaction was carried out under the conditions described in the text. Benzylpenicillin (○) and cefazolin (●) were employed as the substrate at the concentration of 50 μM.



* The method was presented at the 3rd Symposium on "the Molecular Biology of Microorganisms and Its Application to Pharmaceutical Sciences", Nagasaki, Japan, Oct. 28~29, 1976, and at the 36th Kanto Branch Meeting of Japanese Society for Microbiology, Tokyo, Japan, Nov. 18~19, 1976.

Table 1. The outer membrane-permeability of five β-lactam antibiotics and their antibacterial activity.

β-Lactam antibiotics	Conc. of S_1 (μM)	Vd/Vi	Estimated conc. of S_2 (μM)	S_1/S_2	MIC to *P. morganii* 1510/9 (μM)
Benzylpenicillin	8,000	2.2			
	200	258	0.53	377	
	50	406	0.11	455	32
	20	386	0.05	400	
Ampicillin	8,000	1.0			
	200	9	7.5	27	8
	50	20	1.6	31	
	20	73	0.22	91	
Cephalothin	8,000	1.0			
	50	65	0.15	333	32
	20	88	0.08	250	
Cefazolin	8,000	1.2			
	50	9	2.1	24	16
	20	10	1.2	17	
Cephalexin	8,000	1.2			
	50	12	0.97	52	63
	20	22	0.38	53	

the substrate penetrates into the periplasm at a constant diffusion rate after a short lag time (Fig. 1), and that no detectable leakage of the enzyme from the cells suspended in the buffer could be found until 3 hours. The results are shown in Table 1 together with the MIC values of the five β-lactam antibiotics against *P. morganii* 1510/9 which is a cephalosporinase-less mutant derived from *P. morganii* 1510.

The ratio of *Vd* to *Vi* at substrate concentration of 8 mM is the same as "crypticity" which is known as an indicator for the permeability of β-lactam antibiotics through outer membrane of gram-negative bacteria.[4] Our results revealed that this classical crypticity does not reflect the permeability of the antibiotics at their physiological concentrations.

Benzylpenicillin showed a lower permeability than ampicillin. This property is consistent with its characteristics in antibacterial spectrum. However, when the cells are exposed to benzylpenicillin at MIC levels, an expected concentration in the periplasm seems to be the same levels as that of ampicillin, suggesting that there is no significant difference in the activity against the targets between the two penicillins.

Cephalothin showed similar properties to benzylpenicillin in the permeability and the activity against the targets. Other two cephalosporins, especially cefazolin, have a high ability to pass through outer membrane, but activities against the targets seem to be lower.

Although the method presented in this communication includes a non-confirmed prerequisite that β-lactamase expresses about the same enzymatic activity either in the periplasm or in free state, the value S_2 and the ratio of S_1 to S_2 are useful indicators for estimating the fundamental activity against the targets and the outer membrane-permeability of β-lactam antibiotics.

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References

1) KURIYAMA, Y.; M. YAMAMOTO & S. SUGAWARA: β-Lactamase of *Proteus morganii* No. 1510. Seikagaku 46: 413, 1974 (in Japanese)

2) PERRET, C. J.: Iodometric assay of penicillinase. Nature (London) 174: 1012~1013, 1954
3) NOVICK, R. P.: Micro-iodometric assay for penicillinase. Biochem. J. 83: 236~240, 1962
4) RICHMOND, M. H. & R. B. SYKES: The gram-negative bacteria and their possible physiological role. pp. 31~88. *In* A. H. ROSE & D. W. TEMPEST (*ed*), Advances in Microbial Physiology. Vol. 9: Academic Press Inc., London, 1973